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Cloning and Expression of a Complementary DNA Encoding a Molluscan Octopamine Receptor That Couples to Chloride Channels in HEK293 Cells*

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A cDNA encoding a G-protein-coupled receptor was cloned from the central nervous system of the pond snail *Lymnaea stagnalis*. The predicted amino acid sequence of this cDNA most closely resembles the *Drosophila* tyramine/octopamine receptor, the *Locusta* tyramine receptor, and an octopamine receptor (Lym oa_1) that we recently cloned from *Lymnaea*. After stable expression of the cDNA in HEK293 cells, we found that [^{3}H]rauwolscine binds with high affinity to the receptor (K_D = $6.2 \cdot 10^{-9}$ M). Octopamine appears to be the most potent naturally occurring agonist to displace the [^{3}H]rauwolscine binding (K_i = $3.0 \cdot 10^{-7}$ M). Therefore, the receptor is considered to be an octopamine receptor and is consequently designated Lym oa_2. The novel receptor shares little pharmacological resemblance with Lym oa_1, indicating that the two receptors represent different octopamine receptor subfamilies. Octopaminergic stimulation of Lym oa_2 does not induce changes in intracellular concentrations of cAMP or inositol phosphates. However, electrophysiological experiments indicate that octopamine is able to activate a voltage-independent Cl^- current in HEK293 cells stably expressing Lym oa_2. Although opening of this chloride channel most probably does not require the activation of either protein kinase A or C, it can be blocked by inhibition of protein phosphorylation.

G-protein[1]-coupled receptors form a large superfamily of membrane receptors that can be found in all species ranging from unicellular eukaryotes to mammals and that can interact with a large variety of signals (*e.g.* light, odorants, Ca^{2+}, biogenic amines, glycoprotein hormones, etc.) (1).

Bioamines like dopamine, epinephrine, norepinephrine, and octopamine all interact with specific G-protein-coupled receptors. Dopamine is present in high amounts in both vertebrate and invertebrate species. Epinephrine and norepinephrine, on the other hand, are present predominantly in vertebrates, whereas octopamine is considered to act as a major neurotransmitter in invertebrate species only. Octopamine is often referred to as the invertebrate counterpart of norepinephrine because of the structural similarity between these neurotransmitters (they differ only in the presence of a single catecholic hydroxyl group) as well as their functional similarity (both serve an important role in stress adaptation) (2). Consequently, the adrenergic and octopaminergic receptors share pharmacological as well as structural properties (3, 4).

The role of octopamine as a neurotransmitter has been studied particularly well in insects, where it has been shown to interact with at least four different octopamine receptor subtypes (3). The central nervous systems of a number of snails have also been used to study octopaminergic neurotransmission. The presence of octopamine has been demonstrated in the sea slug *Aplysia* (5–7), the land snail *Helix* (8, 9), and the pond snail *Lymnaea* (10). In these species, the interactions of octopamine with its receptors have been described mainly at the electrophysiological level (7, 8, 11–15). In general, application of octopamine induces a hyperpolarization of susceptible snail neurons, a process that is thought to be mediated by an increase in cAMP (2, 11, 12), leading to an increased potassium (12–14) or calcium (15) conductance. In addition, a pharmacological characterization of octopamine receptors has been described for *Lymnaea*.[2]

We have recently cloned and characterized a cDNA encoding an octopamine receptor expressed in the brain of *Lymnaea stagnalis* (16). This receptor (Lym oa_1) shows moderate homology to the *Drosophila* tyramine/octopamine receptor (17, 18), to the *Locusta* tyramine receptor (19) and to the vertebrate α-adrenergic receptors. Activation of this receptor, when expressed in human embryonic kidney (HEK293) cells, leads to elevated concentrations of both intracellular inositol phosphates and cAMP. The pharmacological profile of Lym oa_1 suggests that this receptor represents a previously unknown octopamine receptor subtype.

This paper describes the structure of a second octopamine receptor cDNA (Lym oa_2) cloned from *Lymnaea*. The predicted amino acid sequence of this receptor only shows limited similarity to Lym oa_1, as well as to the insect tyramine receptors and the α-adrenergic receptors. Its pharmacological profile clearly differs from that of previously described receptors. When the novel receptor is stably expressed in HEK293 cells, application of octopamine does not lead to changes in the intracellular concentration of cAMP or inositol phosphates. Acti-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U62770.

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[1] The abbreviations used are: G-protein, guanine nucleotide-binding regulatory protein; HEK293, human embryonic kidney 293 cells; TM, transmembrane region; PCR, polymerase chain reaction; IV, current voltage; PLC, phospholipase C.

[2] S. Juhos, L. Hiripi, M. Eckert, J. Rapus, and K. Elekes, submitted for publication.

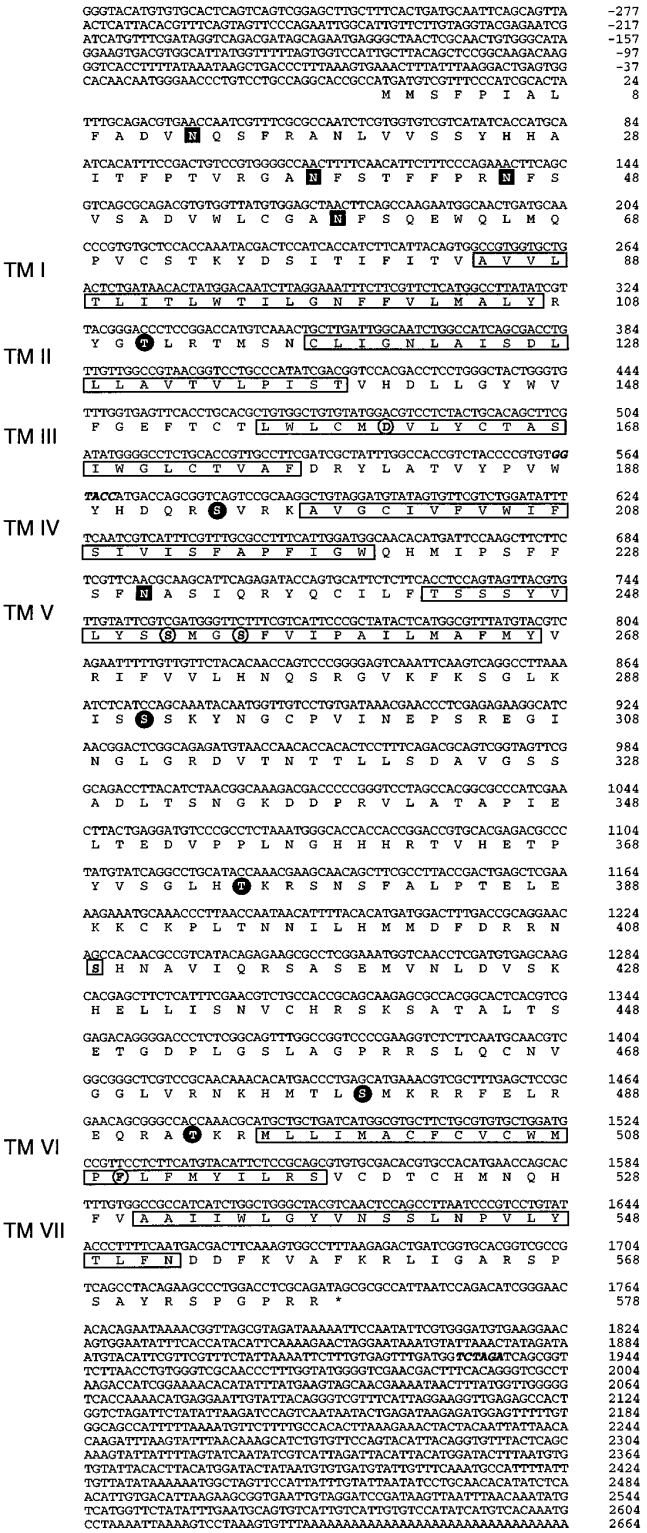

FIG. 1. **Nucleotide and deduced amino acid sequence Lym oa_2 cDNA.** Putative transmembrane regions are *boxed*. Residues in *filled squares* represent consensus sites for *N*-linked glycosylation; residues in *filled circles* represent consensus phosphorylation sites for protein kinase C. Residues in *open circles* have been implicated in bioamine binding. The serine residue in an *open square* can be phosphorylated by protein kinase A. Restriction sites that have been used for the cloning of the expression construct are depicted in *bold italics*.

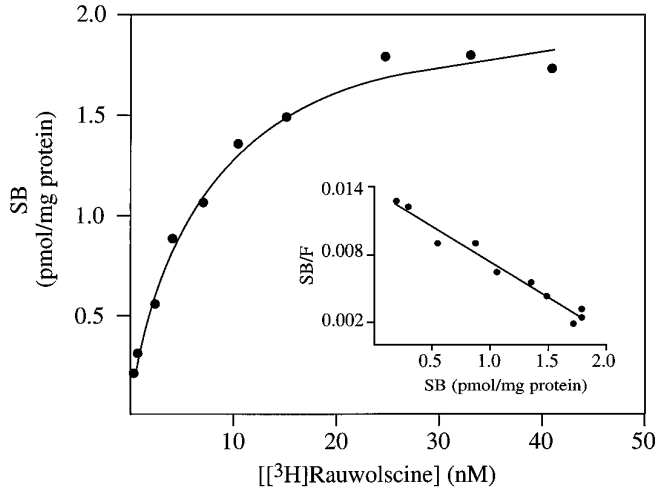

FIG. 2. **Saturable binding of [^{3}H]rauwolscine to Lym oa_2 stably expressed in HEK293 cells.** *Inset*, Scatchard plot of the same data. *SB*, specific binding in picomoles/mg of protein. *SB* represents the total binding minus the nonspecific binding of [^{3}H]rauwolscine to membranes of HEK293 cells expressing Lym oa_2. Nonspecific binding was determined by displacement of [^{3}H]rauwolscine binding by mianserin (5 μM). *F*, free [^{3}H]rauwolscine.

TABLE I

K_i and pK_i values of various compounds for Lym oa_2 as determined by displacement of [^{3}H]rauwolscine binding (6 nM)

K_i values are given (±S.D.) as deduced from three independent experiments, each performed in triplicate. The right column shows the pK_i values of Lym oa_1 (16). These values were obtained in a method similar to that used for Lym oa_2, except for the concentration of [^{3}H]rauwolscine (4 nM).

Compound	pK_i ± S.D.	
	Lym oa_2	Lym oa_1
Agonists		
(±)-*p*-Synephrine	7.01 ± 0.02	6.41 ± 0.24
(±)-*p*-Octopamine	6.52 ± 0.14	5.68 ± 0.18
Xylometazoline	6.10 ± 0.10	5.63 ± 0.03
B-HT 920	5.93 ± 0.20	4.80 ± 0.11
(−)-Norepinephrine	5.71 ± 0.05	4.09 ± 0.39
Clonidine	5.70 ± 0.01	6.57 ± 0.32
(−)-Epinephrine	5.58 ± 0.16	4.26 ± 0.08
p-Tyramine	5.42 ± 0.11	4.44 ± 0.17
Phenylephrine	5.22 ± 0.44	5.60 ± 0.13
Oxymetazoline	5.19 ± 0.05	5.56 ± 0.08
Methoxamine	5.16 ± 0.05	<4
Dopamine	5.05 ± 0.14	<4
Serotonin	4.54 ± 0.21	4.43 ± 0.13
Histamine	4.03 ± 0.18	<4
Antagonists		
Rauwolscine	7.99 ± 0.08	7.46 ± 0.02
Mianserin	7.94 ± 0.25	7.67 ± 0.34
Phentolamine	7.01 ± 0.25	8.07 ± 0.14
Chlorpromazine	6.66 ± 0.28	8.53 ± 0.26
Spiperone	6.31 ± 0.05	8.50 ± 0.31
Yohimbine	6.15 ± 0.09	8.89 ± 0.28
(−)-Propanolol	5.51 ± 0.14	4.93 ± 0.15
Alprenolol	5.42 ± 0.12	5.01 ± 0.41
Prazosine	4.71 ± 0.19	7.02 ± 0.05
Pindolol	4.34 ± 0.46	4.56 ± 0.12

vation of Lym oa_2 does, however, induce a long lasting opening of Cl^- channels. G-protein-mediated activation of a similar Cl^- current was recently described by Postma *et al.* (20) in rat fibroblasts. Their study also indicated that none of the known second messenger system was involved in the Cl^- channel activation. Nevertheless, it was suggested that the increase in chloride conductance was closely associated with the activation of phosphoinositide hydrolysis. In contrast, our results clearly indicate that this novel signaling pathway functions independently from the activation of phospholipase C (PLC). We further show that although protein kinase A and protein kinase C are most probably not involved in the signaling pathway, protein phosphorylation is important for the opening of the Cl^- channel.

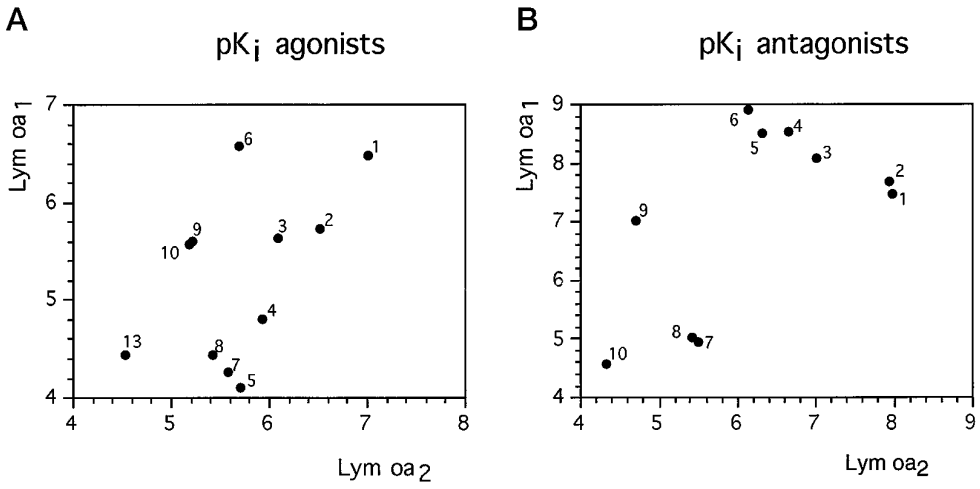

FIG. 3. **Comparison of the pharmacological properties of Lym oa$_1$ and Lym oa$_2$.** *A*, comparison of pK_i values of agonists for both octopamine receptors; pK_i values of <4 are not included in the graph. *B*, comparison of pK_i values of antagonists for both receptors. For binding conditions and numbering of compounds, see Table I.

EXPERIMENTAL PROCEDURES

Isolation of the cDNA Encoding Lym oa$_2$—Central nervous systems of adult *L. stagnalis* bred in the laboratory (21) were dissected, the total RNA was isolated (22), and cDNA was transcribed using oligo(dT) primers and SuperScript™ RNase H$^-$ reverse transcriptase (Life Technologies, Inc.). This cDNA served as a template in a degenerate PCR strategy designed to isolate genes encoding G-protein-coupled bioamine receptors. The oligonucleotide primers recognize stretches of conserved amino acid residues present in transmembrane (TM) regions 6 and 7 and have been described previously (23). PCR products were cloned and sequenced (Sequenase, Life Technologies, Inc.), and one of the fragments revealed significant similarity to the TM6–TM7 region of adrenergic receptors. The presence of several large cDNA clones corresponding to this initial fragment was confirmed in a PCR-based screening of a fractionated *Lymnaea* central nervous system cDNA library in λ-ZAP. Isolated single plaques containing the full-length cDNA inserts were converted into pBS-SK$^-$ phagemids (designated pBS-Lym oa$_2$) by *in vivo* excision, and the insert was sequenced (Sequenase, Life Technologies, Inc.).

Stable Expression of Lym oa$_2$ in HEK293 Cells—A PCR fragment covering the 5′ part of the open reading frame of pBS-Lym oa$_2$ was generated using a sense oligonucleotide based on the DNA sequence around the start codon of the open reading frame and an antisense oligonucleotide based on the sequence 3′ from the endogenous *Kpn*I site (located at position 563; see Fig. 1). This fragment was cloned, sequenced on both strands, and combined with the 3′-coding region of Lym oa$_2$ obtained as a *Kpn*I and *Xba*I fragment (*Xba*I site located at position 1931; see Fig. 1). The total coding region was cloned into pcDNA3 (Invitrogen), yielding a construct designated pcDNA-Lym oa$_2$.

HEK293 cells were stably transfected with pcDNA-Lym oa$_2$ as described before (16). The level of expression of Lym oa$_2$ was determined by measuring the binding of [^{3}H]rauwolscine (81–85 Ci/mmol; Amersham Corp.) to membrane preparations of resistant colonies. One cell line exhibiting a B_{max} of 2.2 pmol/mg was selected for further study.

Radioligand Binding Assays—The preparation of membranes of HEK293 cells and the radioligand binding experiments were carried out as described previously (16).

Saturation isotherms were obtained by incubating membrane protein with increasing amounts of [^{3}H]rauwolscine (0.1–45 nM). Nonspecific binding was determined by the addition of mianserin to a final concentration of 5 μM.

Competition curves were obtained by incubating membrane protein with 6 nM [^{3}H]rauwolscine and increasing amounts of competitor (10^{-10}–10^{-4} M). The obtained data were fitted using Kaleidagraph 3.0 (Abelbeck Software) as described previously (16).

Measurements of Inositol Phosphate Formation—HEK293 cells were grown to ± 50% confluency in 24-well plates, and incubated with 1 μCi of *myo*-[^{3}H]inositol (18 Ci/mmol, Amersham Corp.) per ml of inositol-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) for 20–24 h. Cells were incubated with agonists in the presence of LiCl (10 mM) for 60 min at 37 °C. Cells were lysed with chloroform:methanol, and total cellular inositol phosphates were extracted using Dowex AG 1-X8 anion exchange resin as described previously (16).

Measurements of Cyclic AMP Formation—HEK293 cells were grown to ±80% confluency in 24-well plates and incubated with agonists and 300 μM 3-isobutyl-1-methylxanthine for 20 min at 37 °C. The medium was aspirated and the cells were lysed by sonication in 200 μl of ice-cold 0.1 N HCl. Then, 75 μl of neutralization buffer (230 mM NaOH, 560 mM Tris, 140 mM NaCl, 56 mM EDTA, 0.35 M HEPES) was added, and the concentration of cAMP was determined as described previously (25).

Electrophysiological Experiments—For electrophysiological recordings, HEK293 cells were kept in Petri dishes (Costar) and bathed in 140 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.2, adjusted with NaOH. The composition of standard (non-selective) pipette solution was 135 mM KCl, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, 2 mM MgATP, 0.1 mM Tris-GTP, pH 7.2, adjusted with KOH. To test whether currents were carried by Cl^- ions, KCl in the pipette solution was replaced by K^+-aspartate. Agonists were administered by means of a pico-spritzer (General Valve, Fairfield, U. S. A.) using a small glass pipette (tip diameter 20 μm) placed at a distance of ~100 μm from the recorded cell. This setup enabled rapid application of drugs (≪ 1 s).

Whole cell voltage clamp experiments were performed using either an Axoclamp 2A amplifier (Axon Instruments, Inc., Foster City, CA) in the continuous single electrode voltage clamp mode, in which case cell capacitance (~60 picofarads) was not compensated, or a Liszt EPC7 amplifier (Liszt, Darmstadt, Germany), allowing cell capacitance compensation. Pipettes (2–6 MΩ) were pulled on a Flaming/Brown P-87 (Sutter Instrument Co.) horizontal micro-electrode puller from Clark GC-150 glass (Clark Electromedical Instruments, UK; seal resistance, >2 GΩ). After disruption of the patch membrane, series resistance (<6 MΩ) was compensated for ~70%. Measurements commenced 5 min after access to the cell to allow equilibration with the pipette solution. Data acquisition was controlled by a CED 1400 AD/DA converter (Cambridge Electronics Design, Cambridge, UK) connected to an Intel 80486-based computer run with voltage clamp software developed in our laboratory. The current recordings were filtered at 1–5 kHz, sampled at 1 kHz, and stored on line. This system allowed simultaneous application of voltage steps, acquisition of current recordings, and timed application of drugs.

RESULTS

Cloning of Lym oa$_2$ cDNA—We have applied the PCR technique to clone G-protein-coupled receptors that are expressed in the brain of *L. stagnalis*, using degenerated primers based on highly conserved sequences present in TM6 and TM7 of G-protein-coupled receptors. The obtained PCR products were sequenced and these sequences were conceptually translated. One derived amino acid sequence showed considerable similarity to the sequence of the corresponding region in catecholaminergic receptors. We isolated and sequenced the corresponding full-length cDNA from a *Lymnaea* central nervous system library (see Fig. 1). An open reading frame that can encode a protein of 578 amino acids is present on this cDNA (see Fig. 1).

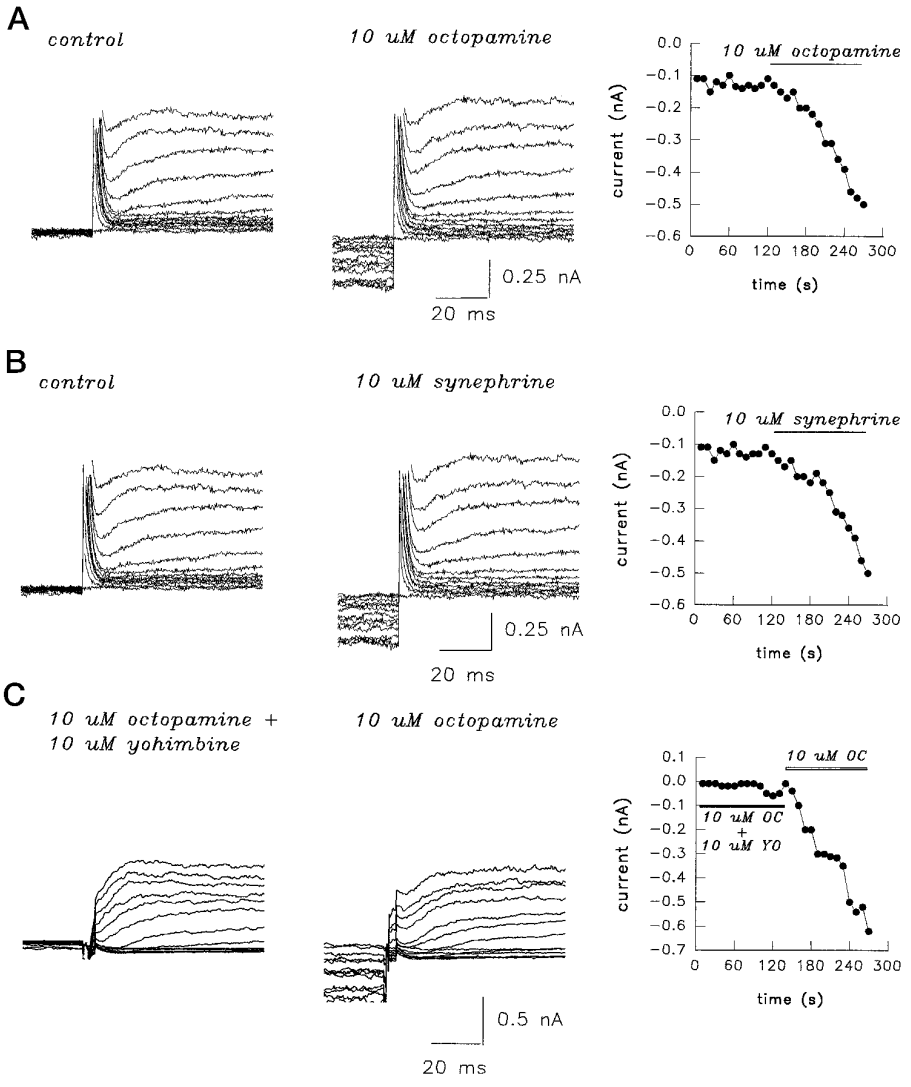

FIG. 4. **Octopamine induces a specific inward current in Lym oa_2-transfected HEK293 cells.** In these whole cell voltage clamp experiments the cells were kept at a holding potential of −80 mV, and voltage steps to test potentials ranging from −50 to +50 mV were given. *Left* and *middle panels* show the holding current preceding each voltage step and the family of responses to the voltage steps of increasing amplitude. In the *right panels* the holding current (measured 10 ms before the onset of the voltage step) is plotted as a function of time. Application of agonist and antagonists is indicated by *horizontal bars*. *A*, octopamine induces a sustained increase in holding current but has little effect on the voltage-dependent outward currents. The current *versus* time plot shows that the inward current response steadily increases during a 3-min application (n = 10). *B*, the octopamine response is mimicked by synephrine (n = 6). *C*, the octopamine response is blocked by yohimbine (n = 3). Subsequent application of only octopamine restores the response. In *A* and *B* cell capacitance was not compensated.

The presence of seven hydrophobic regions characteristic for G-protein-coupled receptors can clearly be recognized within the predicted amino acid sequence. Within these regions, the most prominent amino acid identity is found with the *Drosophila* tyramine/octopamine receptor (49%) (17, 18), the *Locusta* tyramine receptor (50%) (19), Lym oa_1 (45%), and the mammalian α_2-adrenoreceptors (±41%), thus confirming that the open reading frame is likely to code for a bioamine receptor.

Stable Expression and Pharmacological Characterization of Lym oa_2—To further delineate the nature of this receptor we stably expressed it in HEK293 cells. We then used the binding of [^{3}H]rauwolscine to membranes of these cells as a marker to select clonal lines expressing high levels of receptor protein. One clone, exhibiting a B_{max} of 2.2 pmol/mg, was chosen for a pharmacological characterization of the receptor. Fig. 2 shows the saturation binding curve and the corresponding Scatchard plot of the binding of [^{3}H]rauwolscine. The affinity constant (K_D) of [^{3}H]rauwolscine for the novel receptor is 6.24 nM. The ability of several (mainly adrenergic) compounds to displace [^{3}H]rauwolscine binding is presented in Table I. The rank order of potencies of selected agonists is: (±)-*p*-synephrine > (±)-*p*-octopamine > xylometazoline > (−)-norepinephrine = clonidine > epinephrine ≥ *p*-tyramine > dopamine. The rank order of potencies of selected antagonists is: rauwolscine = mianserin > phentolamine > spiperone > yohimbine > (−)-propanolol > prazosine > pindolol. Since octopamine is the most potent naturally occurring agonist to displace the [^{3}H]rauwolscine binding, the receptor was considered to be an octopamine receptor and consequently called Lym oa_2.

We recently cloned and characterized another octopamine receptor from *Lymnaea* (Lym oa_1; Ref. 16). To allow for a comparison of the binding properties of both octopamine receptors, Table I also shows the pK_i values of the same set of ligands obtained by the displacement of [^{3}H]rauwolscine binding from Lym oa_1. As can be seen from a Pearson correlation graph (Fig. 3) the pharmacological profiles of Lym oa_1 and Lym oa_2 are considerably different.

Signal Transduction of Lym oa_2—We tested to discover to which signal transduction pathways Lym oa_2 can be coupled. In transiently transfected HEK293 cells, as well as in four independently isolated stable cell lines, octopaminergic stimulation did not induce any change in the concentration of cAMP or inositol phosphates. Also, stimulation with tyramine, epinephrine, norepinephrine, dopamine, serotonin, or histamine did not change the concentration of these second messengers as compared with non-transfected HEK293 cells. Positive control experiments, however, resulted in pronounced increases in cAMP or inositol phosphates (stimulation of endogenous β-adrenergic receptors increased cAMP 20-fold over basal levels, and stimulation of Lym oa_1 receptors expressed in HEK293 cells increased inositol phosphates 45-fold over basal levels; data not shown).

Since activation of many neuronal receptors induces changes in membrane conductances of the cells in which they are ex-

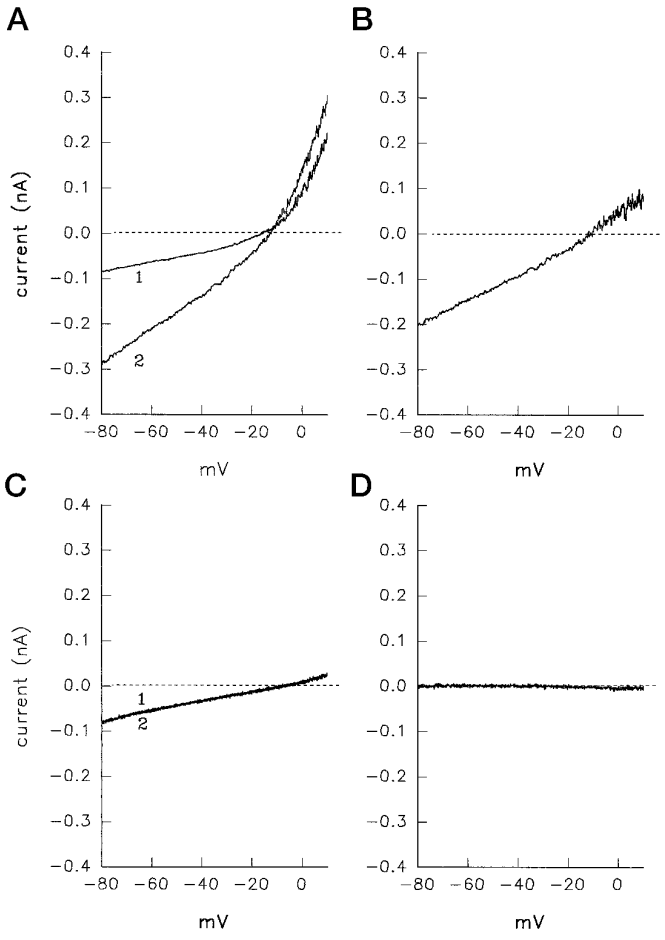

FIG. 5. **Octopamine activates a voltage-independent Cl^- current.** Current responses were recorded in experiments in which voltage ramps were applied. Over a period of 8 s the voltage was continuously varied from −80 to +10 mV. *A*, current responses in the absence (*1*) and presence (*2*) of octopamine using standard salines with $[Cl^-]_{out}$ = 148.8 mM and $[Cl^-]_{in}$ = 137 mM, yielding a theoretical reversal potential for Cl^- of −2 mV. *B*, current voltage relation of the isolated current response to octopamine, obtained by subtraction of trace *1* from trace *2* from *panel A*. Reversal of the current response is close to the theoretically expected potential (n = 6). *C* and *D*, like *A* and *B*, but with internal Cl^- replaced by aspartate, thus shifting the Cl^- reversal potential to −108 mV. Note the control response and the response in the presence of octopamine overlap, indicating that, with aspartate in the cell, the current response is eliminated (n = 5).

pressed, we tested whether application of octopamine to HEK293 cells expressing Lym oa_2 elicited electrical responses in these cells. Application of 10 μM octopamine to HEK293 cells expressing Lym oa_2 induced a large but slow increase in the holding current of the voltage-clamped cells (Fig. 4*A*). The amplitude of the octopamine-induced current decreased at more depolarized potentials. Application of synephrine (10 μM), an agonist with a higher potency than octopamine (see Table I), mimicked the octopamine-induced response, whereas yohimbine (10 μM), an antagonist, inhibited the response, thus confirming the specificity of the effect (see Fig. 4, *B* and *C*). Application of octopamine to non-transfected HEK293 cells did not show any effect (Fig. 7*A*).

In order to study the nature of the current (*e.g.* the voltage dependence and ionic selectivity), we performed voltage ramp experiments. Current responses were recorded while the voltage was continuously varied from −80 mV to +10 mV over a period of 8 s, both in the absence and presence of octopamine (Fig. 5*A*). The current voltage (IV) relation of the octopamine-induced current was obtained by subtracting the control current from the current response in the presence of octopamine. Fig. 5*B* shows that octopamine activates a current response over the whole voltage range tested and that the IV relation of the octopamine-induced current is almost linear, indicating that the current is voltage-independent. Comparison of the IV relations of nonstimulated and stimulated cells revealed no or only minor differences (<5 mV) in reversal potential (Fig. 5*A*). The IV curve of the octopamine-induced current (isolated by subtraction) showed reversal around −10 mV, which is close to the reversal potential of chloride ions (E_{Cl-} = −2 mV under the present ionic conditions). To test whether the octopamine response is indeed carried by Cl^- ions, we replaced KCl in the intracellular (pipette) medium with K^+-aspartate, thus shifting E_{Cl-} to −108 mV. This change caused a dramatic loss of the octopamine-induced inward current response (Fig. 5, *C* and *D*). While under standard Cl^- conditions (measured at −90 mV) octopamine induced an increase in inward current of 149 ± 33 pA (n = 6), this reversed to a negligible outward current of 13 ± 7 pA (n = 5) with aspartate replacing Cl^- in the pipette. This result strongly suggests that octopamine activates chloride channels.

We then performed a limited number of initial experiments to study the signal transduction pathway underlying the activation of the Cl^- channel. The above experiments already indicated that the process is slow; the current still increased 10 min after application of octopamine. To see whether a phosphorylation step is involved, we tested the effect of the nonspecific protein kinase inhibitor H1004 on the octopamine-induced current. Fig. 6 shows that in the presence of 50 μM H1004, octopamine did not induce or only very slightly induced the inward Cl^- current, whereas subsequent application of only octopamine did evoke the normal response.

Interestingly, we observed that HEK293 cells stably expressing Lym oa_1 showed the same current response to octopamine as cells expressing Lym oa_2 (Fig. 7*B*). The effect, however, is not a general consequence of the activation of overexpressed, heterologous receptors, since stimulation of a *Lymnaea* serotonin receptor (5-HT_{2Lym}) stably expressed in HEK293 cells (23) did not show any effect on the Cl^- conductance (Fig. 7*C*). Furthermore, stimulation of β-adrenergic receptors that are endogenously present in HEK293 cells did not influence the inward current (Fig. 7*D*). Stimulation of Lym oa_1, Lym oa_2, 5-HT_{2Lym}, and β-AR all differentially influenced the outward current. These effects, however, were not studied in detail.

DISCUSSION

We have used a degenerate PCR strategy to isolate cDNAs encoding G-protein-coupled bioamine receptors that are expressed in the central nervous system of the pond snail *L. stagnalis*. Recently, we reported the cloning and expression of a serotonin receptor (5-HT_{2Lym}; Ref. 23) and an octopamine receptor (Lym oa_1; Ref. 16) using the same strategy. This paper describes the isolation of a cDNA encoding a second octopamine receptor, designated Lym oa_2. The predicted amino acid sequence of Lym oa_2 exhibits the highest similarity to the *Drosophila* tyramine/octopamine receptor, the *Locusta* tyramine receptor, and Lym oa_1, and to the vertebrate α-adrenergic receptors. Although both Lym oa_2 and Lym oa_1 encode *Lymnaea* octopamine receptors, their amino acid identity is only moderate, *i.e.* 45% in the TM regions. An interesting difference in the sequence of both octopamine receptors can be found in TM5. Mutagenesis studies on catecholaminergic receptors have indicated that two conserved serine residues in this domain (Ser^{204} and Ser^{207} in the β-adrenergic receptor) play a crucial role in ligand binding. Supposedly, the two Ser hydroxyl groups can hydrogen bond to the catechol hydroxyl groups of the ligand (see for instance Refs. 26–28). In octopamine receptors there is

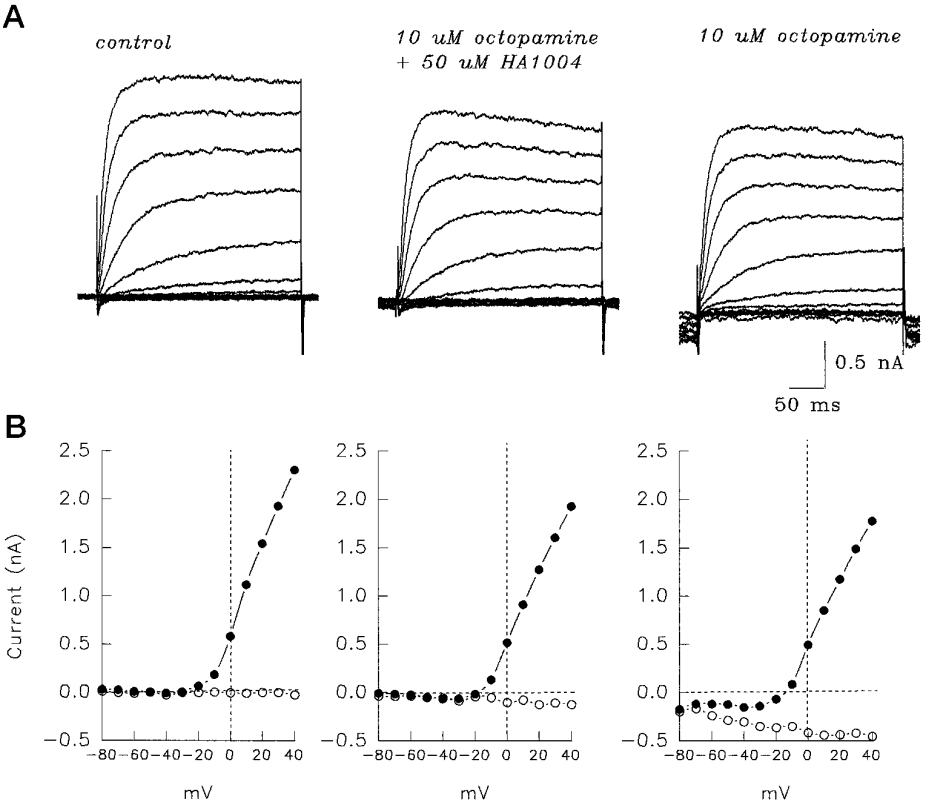

FIG. 6. **Blocking effect of HA1004 on the octopamine response.** *A*, voltage clamp experiments as in Fig. 4. *Left panel*, control. *Middle panel*, in the presence of the protein kinase blocker HA1004, octopamine only induces a minimal inward current response. *Right panel*, normal current response when only octopamine is applied. *B*, current response amplitude *versus* test pulse potential during control conditions, during octopamine and HA1004 application, and during application of only octopamine, as indicated (n = 3). *Filled circles*, maximal outward current amplitude. *Open circles*, amplitude of the holding current, measured 10 ms before onset of the responses to the voltage steps.

no obvious need for conservation of both serine residues in TM5 because the aromatic ring of octopamine is monohydroxylated. Indeed, in Lym oa_1 only a single serine is found at the relevant position in TM5. In Lym oa_2, however, both serines are present. Interestingly, the catecholamines (epinephrine, norepinephrine, dopamine) show a considerably higher affinity for Lym oa_2 than for Lym oa_1 (see Table I). This suggests that the serine residues in TM5 play a role in agonist binding in the *Lymnaea* octopamine receptors similar to their role in the vertebrate catecholamine receptors.

In general, agonists exhibit higher affinities for Lym oa_2 than for Lym oa_1, while antagonists have higher affinities for Lym oa_1 than for Lym oa_2. Another interesting difference between the binding properties of both octopamine receptors is the opposite order of affinities for the isoschizomers rauwolscine and yohimbine. Lym oa_1 has a higher affinity for yohimbine than for rauwolscine (pK_i = 8.9 *versus* 7.5), while Lym oa_2 has a higher affinity for rauwolscine than for yohimbine (pK_i = 8.0 *versus* 6.2). Both the pharmacological profiles of Lym oa_1 and Lym oa_2 indicate a closer relationship to the α-adrenergic receptors than to the β-adrenergic receptors. In that respect it is noteworthy to mention the much higher affinity of the α_1 antagonist prazosine for Lym oa_1 (pK_i = 7.0) than for Lym oa_2 (pK_i = 4.7). Whereas the pharmacological profile of Lym oa_1 still shows a moderate similarity to that of the *Drosophila* tyramine/octopamine receptor, the *Locusta* tyramine receptor, and the α_2-adrenergic receptors, the pharmacological profile of Lym oa_2 clearly differs from that of Lym oa_1, the tyramine receptors, the adrenergic receptors, and the insect octopamine receptor subtypes as described in tissue preparations.

Stimulation of Lym oa_2 did not lead to the activation of the classical signal transduction pathways mediated by adenylyl cyclase and PLC. The receptor is, however, clearly able to transduce signals since we found that HEK293 cells expressing Lym oa_2 showed octopamine-induced changes in their membrane conductance. More specifically, we observed a slow but large increase in the holding current of voltage-clamped HEK293 cells upon application of octopamine. At potentials below the Cl^- equilibrium potential, this response was observed as an increase in inward current, most likely caused by an efflux of Cl^- ions. The process underlying the opening of the Cl^- channels may involve protein phosphorylation, since the (nonselective) protein kinase inhibitor HA1004 inhibited the inward current. Alternatively, the Cl^- channels involved may need to be in a phosphorylated state to be able to open in response to the octopamine stimulus. Because the IV relationship of the current response proved to be linear and because no changes in inositol phosphates were observed upon octopamine application, the Cl^- channels involved are suggested to be both voltage-independent and Ca^{2+}-independent. Additional effects of octopamine on (voltage-dependent) outward currents were also observed but were not pursued in detail.

Interestingly, stimulation of HEK293 cells expressing the other *Lymnaea* octopamine receptor, Lym oa_1, resulted in a similar outward Cl^- current. In contrast to Lym oa_2, Lym oa_1 has previously been shown to activate both PLC and adenylyl cyclase. To examine whether the effect on the Cl^- channel might be secondary to the effects on adenylyl cylase and PLC or might represent an independent signaling route, we tested the effect of activation of two other receptors, expressed in HEK293 cells. Stimulation of these receptors, *i.e.* a *Lymnaea* serotonin receptor or an endogenous β-adrenergic receptor, led to activation of PLC and adenylyl cyclase, respectively. The subsequent rise in levels of diacylglycerol or cAMP will activate protein kinase C or protein kinase A, respectively. Stimulation of these receptors did, however, not show any effect on the inward current, suggesting that protein kinase C or protein kinase A is not involved in the phosphorylation process underlying Cl^- channel activation.

It remains to be investigated whether stimulation of Lym oa_2 in *Lymnaea* neurons will also affect the Cl^- conductance. Activation of octopamine receptors on neurons of other snails has been described to lead to outward potassium currents (12–14) and inward calcium currents (15). Early studies on *Aplysia*

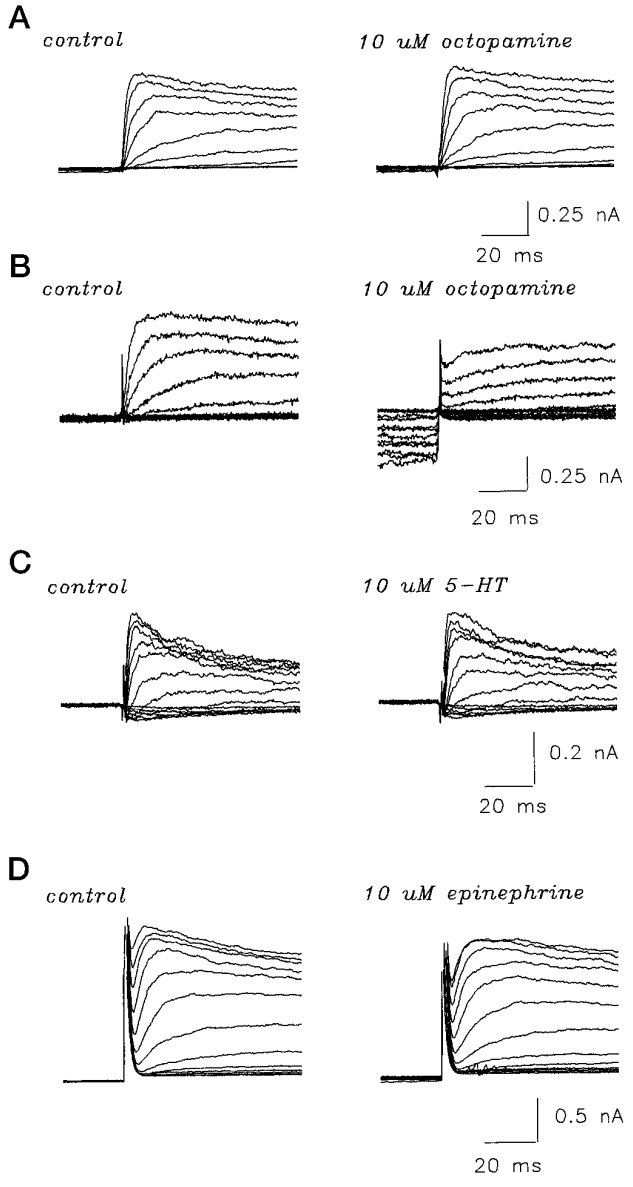

FIG. 7. **Effect of stimulation of other receptors on the inward current, observed as an increase in the holding current preceding the responses to a voltage step.** *A*, application of octopamine to non-transfected HEK293 cells fails to elicit a response ($n = 3$). *B*, application of octopamine to HEK293 cells expressing Lym oa_1 induces a similar response as in Lym oa_2-transfected cells ($n = 5$). *C*, application of serotonin to HEK293 cells expressing $5\text{-}HT_{2Lym}$ fails to elicit a similar response ($n = 3$). *D*, application of epinephrine to non-transfected HEK293 cells, endogenously expressing β-adrenergic receptors, also fails to elicit a similar response ($n = 2$).

octopamine receptors (29) have shown that stimulation of neurons with octopamine induces the specific phosphorylation of a particular unidentified protein. In this case, however, it was suggested that the process might involve protein kinase A, since elevating the intracellular concentration of cAMP produced a similar effect.

Recently, it was shown that activation of the G-protein-coupled lysophosphatidic acid receptor present on fibroblasts leads to a long lasting depolarization of these cells due to an efflux of chloride ions (20). As was found in our studies, the signal transduction pathway responsible for opening the chloride channel was suggested to be independent of known second messengers. The opening of this particular chloride channel could also be induced by activation of the thrombin receptor, the endothelin receptor, and the neurokinin A receptor. Since these receptors (as well as the lysophosphatidic acid receptor) all couple to PLC, it was suggested that the signaling pathway leading to the increased Cl^- conductance is closely associated with phosphoinositide hydrolysis (20).

All available data suggest that the chloride channel in HEK293 cells that we have found to be G-protein-activated is highly similar to the channel described by Postma *et al.* (20). Our data, however, exclude the option that phosphoinositide hydrolysis is important in the signaling pathway leading from the activated receptor to the opening of the chloride channel, since stimulation of Lym oa_2 does not result in any change in intracellular concentrations of inositol phosphates. Also, activation of a serotonin receptor that has been shown to be coupled to the activation of PLC does not influence the outward Cl^- current. The G-protein-mediated activation of the chloride channel described by Postma *et al.* in Rat-1 fibroblasts (20) and by ourselves in HEK293 cells must proceed via an as yet unknown signaling pathway. Here we show that protein phosphorylation is important in this pathway but that protein kinase A and protein kinase C are not involved. The identification of the relevant kinase and the further examination of the signal transduction pathway will be the topics of future investigations.

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